New three-stage *in vitro* model for infant colonic fermentation with immobilized fecal microbiota

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Abstract

The development and validation of a new three-stage culture system with immobilized fecal microbiota to simulate infant colonic ecosystem is described. Two continuous cultures with different fecal inocula were used to assess the validity and stability of the intestinal model. The total anaerobe populations measured in beads and effluent fermentations reached high concentrations similar to infant feces. Fluorescence *in situ* hybridization analyses and denaturing gradient gel electrophoresis profiles of effluent samples from the three reactors revealed complex patterns similar to that observed in the inoculum, indicating that fecal bacterial diversity was well-preserved and that dominant bacterial populations showed good stability among reactors. For both experiments, the bacterial populations and fermentation product concentrations were in the range of published data for infant feces. These results demonstrate that this new three-stage continuous culture with immobilized cells provides a useful tool for studying the infant colon ecosystem.

Introduction

Different *in vitro* systems, which can be divided into static batch and continuous cultures, have been applied to study the human colonic microbiota (Rumney & Rowland, 1992).Whereas batch cultures are better for short-term studies on metabolism, continuous cultures are more suited for long-term fermentation studies to investigate composition and metabolic activities of the colonic microbiota under the influence of certain factors and to simulate *in vivo* conditions. Different levels of complexity, from a single culture vessel to three vessels in series representing the proximal, transverse and distal colons, have been studied (Rumney & Rowland, 1992; Minekus *et al.*, 1999). However, these conventional continuous culture systems, inoculated with diluted feces from one or several donors, present several limitations due to the free-cell state of their bacterial populations (Cinquin *et al.*, 2004). Indeed, they may not be fully representative of the complex bacterial community which is present both at planktonic and sessile states in the colon (Macfarlane *et al.*, 1997). Moreover, they often show lower cell density ($<10^9$–10^{10} CFU mL^{-1}) (Probert *et al.*, 2004) compared with colonic contents (10^{10}–10^{11} CFU mL^{-1}) as well as limited time stability. The wash-out of less competitive bacteria in the complex fecal microbiota during the extended period (*ca.* 2 weeks) needed to stabilize the culture may also occur with free cell systems (Macfarlane *et al.*, 1998a).

In a previous study, we developed a new *in vitro* infant colonic model composed of a single-stage culture with immobilized infant feces (Cinquin *et al.*, 2004). Using microbiological methods (plate counts on selective media) we showed that a fecal microbiota could be successfully immobilized while preserving major bacterial populations for 54 days of continuous culture. Very good stability and high cell density were reached in beads and in fermentation effluents as a result of immobilized fecal microbiota growth, cell release from beads and eventually growth of free cells in the bulk medium. However, single-stage culture systems can only accurately model the proximal colon (Cinquin *et al.*, 2004). In order to improve our first model using immobilized infant fecal microbiota, in this study we developed and tested a three-stage chemostat model, composed of three reactors in series, simulating simultaneously the proximal, transverse and distal parts of the infant colon. This model was validated by two continuous culture experiments carried out for a total of 29 and 11 days, with different infant fecal inocula.

Several nutritive media with total carbohydrate concentrations ranging from 7.4 to 21 g L^{-1} have been designed to simulate adult ileal chyme in different *in vitro* colonic models (Minekus *et al.*, 1999; McBain & MacFarlane, 2001). In this study, a medium with two different total carbohydrate concentrations (13 and 10 g L^{-1}) was tested to assess the effects of this composition parameter on bacterial populations and metabolic activity within the same continuous culture experiment and during two separate experiments with different fecal inocula.

In our first study (Cinquin *et al.*, 2004) we used plate counts on selective media to monitor bacterial populations. Since cultivation methods are insufficient to analyze the complex microbiota of the human gut accurately, fluorescence *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) were also used to assess population counts and bacterial diversity in the immobilized cell colonic model. Indeed, the application of molecular tools in numerous studies has confirmed an abundance of microbes that cannot be recovered with selective plating of intestinal samples and the need for culture independent methods to characterize the intestinal microbiota composition (Suau *et al.*, 1999).

Materials and methods

Fecal samples collection and preparation

Fecal samples used for immobilization were collected from two healthy 6-month-old infants, who had never received antibiotics and were in the weaning period, and were maintained in anaerobiosis as described by Cinquin *et al.* (2004). Both infants had milk as major dietary component, with some cereals and legumes introduced during the fifth month of life. However, baby 1 had been fed with infant formula after the third month, whereas baby 2 received only breast milk. Samples of fecal suspensions were collected for metabolites, plate counts, FISH and DGGE analyses. The procedure was completed within 2 h of defecation.

Immobilization technique

The fecal inocula were immobilized in 1–2 mm diameter gel beads, composed of 2.5% gellan gum, 0.25% xanthan gum and 0.2% sodium citrate (volume in weight, v/w). The immobilization procedure was based on a dispersion process in a two-phase system, as described by Cinquin *et al.* (2004). The entire process was completed under aseptic conditions within 1 h.

Fermentation media

The fermentation medium used to simulate the ileal chyme produced by an infant diet was based on the composition described by Cinquin *et al.* (2004) and contained (g L^{-1} of distilled water): bile salts (0.05), mucin (4), yeast extract (2.5), hemin (0.01), Tween 80 (1), salts (NaCl, 4.5; KCl, 4.5; $MgSO_4$ $7H_2O$, 1.25; $CaCl_2$, $2H_2O$, 0.15; K_2HPO_4, 0.5; $NaHCO_3$, 1.5; $FeSO_4$ $7H_2O$, 0.005), vitamins and cystein (0.8). The compositions of nitrogen compounds and carbohydrates were calculated from the composition of a standard infant formula supplemented with rice starch (Sigma, St Louis, MO) and applying digestibility indices from the literature. The total concentration of nitrogen compounds (casein, whey protein hydrolysate, peptone and tryptone) was 9.4 g L^{-1}. Two different total carbohydrate concentrations (10 and 13 g L^{-1}), sufficient to sustain good bacterial growth but with different effects on bacterial activity (Macfarlane *et al.*, 1998b), were tested to determine which one was the most appropriate to mimic the infant ileal chyme. The ratio of the different carbohydrates in the media remained unchanged, with 7% soluble rice starch, 18% lactose and 75% maltodextrin in both experiments.

Fermentation procedures

Batch fermentations were first carried out for inoculated bead colonization in a custom-stirred flat bottom glass bioreactor (R1: working volume 100 mL) containing 30% (volume in volume, v/v) of freshly prepared beads (Cinquin *et al.*, 2004). This reactor was then used as the first stage reactor with immobilized cells in the three-reactor model. For colonization, the fermented medium was aseptically replaced by fresh medium every 12 h for a total fermentation period of 48 h (Cinquin *et al.*, 2004). After colonization, two reactors (R2 and R3), half-filled with sterile fermentation medium, were connected in series to R1. Reactor R1 was continuously fed with fermentation medium using an accurate low flow rate produced by a peristaltic pump (Minipuls 3, model M312, Gilson, Villiers le Bel). The fermented medium from R1 was transferred in R2 (working volume 125 mL) and R3 (working volume 100 mL) with a multi-head peristaltic pump (Multistatic pump, model 426–2000, Buchler Instruments, Lenexa, KS). The fermentation volume in each reactor was controlled by adjusting the height of the harvesting tube.

The fermentation conditions applied (retention time and pH) in R1, R2 and R3 were selected to simulate proximal (PCS), transverse (TCS) and distal (DCS) infant colons (Sievers *et al.*, 1993; Fallingborg, 1999). The total mean retention time in the system with a total fermentation volume of 325 mL was set at 13 h by adjusting the feed flow rate to 25 mL h^{-1}, for mean retention times of 4, 5 and 4 h in R1, R2 and R3, respectively. Mixing in each reactor was performed with a magnetic stirrer, and the temperature was controlled at 37 °C by circulating thermostated water in the reactor double wall. Sterile CO_2 was sparged in the liquid

medium of all fermentation vessels. The pH was controlled at 5.9 and 6.2 in R1 and R2, respectively, with two pH-meter titrators (PHM84, TTT80 Titrator, Radiometer, Copenhagen) connected to magnetic valves that dispensed NaOH (6 N). The pH in R3 was not controlled but stabilized at the physiological range of 6.6–6.7 (Fallingborg, 1999).

Two experiments were carried out using fermentation media with either 13 or 10 g L^{-1} total carbohydrate concentrations. The first fermentation, F1, which lasted 29 days, included a colonization (48 h of batch cultures) and a stabilization period (12 days), followed by a period testing the effect of FOS (fructooligosacharide, Nutraflora® P-95, Canacure, Laval, Quebec) substituting maltodextrin in the medium on infant microbiota (8 days: data not shown). All these fermentation steps (total period of 22 days) were applied, with a total carbohydrate concentration in the medium of 13 g L^{-1}. Then for the next 7 days, the system was re-stabilized with the fermentation medium containing 10 g L^{-1} of carbohydrate. For the second fermentation, F2, a fermentation medium with 10 g L^{-1} of total carbohydrate concentration was used during colonization (48 h) followed by a stabilization period of 9 days. An effluent sample (4 mL) was collected daily in each reactor for metabolites, plate counts, DGGE and FISH analyses. Bead samples (0.5–1 g) were also collected after immobilization and 48 h colonization and during pseudo-steady-state periods for plate counts and FISH analyses. The pseudo-steady state was reached when bacterial populations in the fermentation effluents from all reactors did not change by more than 0.5 Log10 unit, this corresponding to the last four days of each culture experiment.

Bacterial enumeration with plate counts

All plate counts were performed within 1 h of sampling with selective and nonselective media as described previously (Cinquin *et al.*, 2004). Cell counts were performed in duplicate and expressed as Log10 CFU per g (wet weight) of feces, per mL of fermentation medium, or per g (wet weight) gel beads. Bacterial survival rates in freshly prepared beads were measured as described by Cinquin *et al.* (2004), and expressed with respect to initial counts in fecal samples (Log10 CFU g^{-1} wet feces).

Bacterial enumeration with FISH

Fluorescence *in situ* hybridization analyses were performed as described by Schwiertz *et al.* (2000) on fixed fecal supernatants (1.5 mL) and fermentation samples (1.5 mL) from the last 2 days of the pseudo-steady-state periods in both fermentations. Staining with 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg/mL was used to measure total cell counts. Different oligonucleotide Cy3-labeled probes, with hybridization conditions (lysozyme treatment, buffers and hybridization temperatures) specific for each probe as described in probe references, were used to detect the main populations: Eub338 for total bacteria (Amann *et al.*, 1995), Bac303 for bacteroides (Manz *et al.*, 1996), Erec482 for *Clostridium coccoides–Eubacterium rectale* group (Franks *et al.*, 1998), Bif164 for bifidobacteria (Langendijk *et al.*, 1995), Lab158 for *Lactobacillus* and *Enterococcus* (Harmsen *et al.*, 1999), and Ec1531 for *Escherichia coli* (Poulsen *et al.*, 1995). To prevent fading of fluorescence, the slowfade® antifade kit was used (Molecular Probes, Eugene, OR). Cells were counted visually with an Olympus BX 51 epifluorescence microscope (Olympus America, Melville, NY). A radial cell concentration gradient is typically observed in wells. To avoid the counting error due to this specific distribution, the bacterial concentration was calculated from the bacterial density of annular regions. To accurately estimate probe-hybridized cell numbers, each well (diameter of 6000 μm) was subdivided into 127 μm thick annular regions, giving a total of 15 regions. To estimate cell numbers in each annular region, the objective of the microscope was first positioned on the geometric center of the well and then moved manually, with a first displacement of 73 μm, and afterwards by increments of 127 μm. The number of bacteria counted for opposite fields from the center of the well was used to estimate the total number of bacteria in the annular region i (N_{Ai}) using the following formula: $N_{Ai} = N_{Fi} \times S_{Ai}/S_F$, where N_{Fi} is the mean cell number calculated for the two fields representing the annular region i, and S_F and S_{Ai} are the surface of the field (73 μm × 42 μm) and of the annular region i, respectively. The total cell number in a well was then calculated by adding the cell numbers obtained from each of the 15 annular regions comprising the well. Each assay was carried out in duplicate.

Hybridization and microscopic observation of beads

Fluorescence *in situ* hybridization was used to visualize bacterial distribution of total anaerobes (Eub338), bifidobacteria (Bif164), *Bacteroides–Prevotella* cluster (Bac303), *Clostridium coccoides–Eubacterium rectale* group (Erec482) and *Lactobacillus-Enterococcus* (Lab158) within gel beads sampled during pseudo-steady-state periods of both fermentations. Beads used for FISH analyses were frozen with 20% glycerol and stored at −80 °C. After thawing they were washed several times with phosphate-buffered saline (PBS) before being fixed with one volume of PBS and three volumes of paraformaldehyde 4% (weight in volume, w/v). After one night at 4 °C, they were cut into two parts with a razor blade, washed twice in PBS and kept at 4 °C for no more than 2 days. In order to preserve the bead gel structure, hybridizations with different Cy3-labeled probes were

carried out overnight at low temperature of 35 °C with 30% of formamide, which is sufficient to keep probe specificity as shown by Rigottier-Gois *et al.* (2003). After hybridization, beads were stored in PBS and kept in a cold dark room for a maximum of 4 days. Hybridized half-beads were placed on to cover slides (the cut surface facing the cover slide) mounted on 1 mm-thick concave microslides (Prioult *et al.*, 2000). Microscopic observations were performed by confocal-laser scanning microscope (LSM 310; Carl Zeiss, Oberkochen) equipped with an He–Ne laser (543 nm) as the excitation source, and with a photomultiplier that selected emission signals from 575 to 640 nm. For each period and probe tested, five to 10 beads were analyzed.

DNA isolation, PCR and DGGE analysis

After homogenization and centrifugation, 1.5 mL of fecal inoculum supernatant was centrifuged at 10 000 ***g*** for 10 min. The supernatant was then removed and the bacterial pellet was stored at −80 °C until its use. DNA extraction of fecal and fermentation samples was performed using the FastDNA SPIN kit (Qbiogene, Carlsbad, CA). Primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 (Nubel *et al.*, 1996) were used to amplify the V6 to V8 regions of the 16S rRNA gene as described by Konstantinov *et al.* (2004). PCR products were separated by DGGE based on the protocol of Muyzer *et al.* (1993) using a Bio-Rad system (Bio-rad, Hercules, CA). The polyacrylamide gel used was composed of 8% (v/v) polyacrylamide and 1× Tris acetate EDTA (TAE) buffer. The polyacrylamide gel was made with a denaturing gradient ranging from 30% to 60% (100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 h at 85 V and 60 °C. After the electrophoresis, the gel was stained with $AgNO_3$.

Metabolites analyses

Short-chain fatty acids (SCFA: acetate, propionate, butyrate and valerate), isoacids (isobutyrate and isovalerate) and lactate were determined by high-performance liquid chromatography (HPLC) analysis (Waters, Milford, MA) equipped with Agilent ChemStation Version 6.2 and a differential refractometer (Model R410, Waters) as previously described by Cinquin *et al.* (2004). Ammonia concentration in feces and effluent fermentation samples was measured with an ammonia electrode (VWR, West Chester, PA). Each analysis was done in duplicate. The mean metabolite and ammonia concentrations after stabilization were calculated for the last 3 days of pseudo-steady-state periods and expressed in mM. The metabolite and ammonia concentrations in feces were expressed in mmol kg^{-1} of wet weight feces.

Statistical analysis

A two-way analysis of variance (ANOVA) was performed with JMPIN® (SAS Institute Inc., Cary, NC) to test the effects of the detection method (plate counts selective media vs. FISH) and reactor stage on bacterial populations. A one-way ANOVA was used to test the effects of the reactor stage and carbohydrate concentration (for F1) on metabolite production during the last 3 days of fermentation. When significant differences were found below the probability level of 0.05, treatment means were compared using the Tukey–Kramer-HSD test.

Results

Immobilization of fecal samples and bacterial diversity in beads

Fecal samples from the two babies, used for immobilization for F1 and F2, showed similar concentrations of total and facultative anaerobes but different population profiles (Table 1). Compared with that of baby 1 (F1), the fecal microbiota of baby 2 (F2) was dominated by bifidobacteria (10.3 vs. 9.5 Log10 CFU g^{-1}) with higher counts of Gram-positive cocci (9.1 vs. 7.9 Log10 CFU g^{-1}), staphylococci (7.5 vs. 5 Log10 CFU g^{-1}) and lactobacilli (8.2 vs. <4 Log10 CFU g^{-1}), but lower counts of *Bacteroides* spp. (8.9 vs. 9.6 Log10 CFU g^{-1}) and clostridia (6.7 vs. 8.1 Log10 CFU g^{-1}). Immediately after immobilization, the main bacterial populations tested were recovered in beads, except for staphylococci in F1, which were already close to the detection limit of the method (<4 Log10 CFU g^{-1} feces) in the fecal inoculum (Table 1). The bacterial survival rates for the different populations ranged between 1% and 40% with lowest values for lactobacilli (1%) and *Bacteroides* spp. (1–6%).

At the end of the initial stabilization periods (14 and 11 days for F1 and F2, respectively), beads were highly colonized with total anaerobe concentrations reaching 10.4 and 10.0 Log10 CFU g^{-1} of beads for F1 (13 g L^{-1} of total carbohydrates) and F2 (10 g L^{-1} of total carbohydrates), respectively (Table 1). In fermentation F1, a reduction in carbohydrates from 13 to 10 g L^{-1} decreased the total and facultative anaerobe count by *ca.* 1 Log10 unit. A decrease in bacterial counts (in the range of 0.4–2.5 Log10 CFU g^{-1} beads) was generally observed for the main bacterial populations tested, except for lactobacilli and clostridia which increased by 0.5 and 0.9 Log10 CFU g^{-1} beads, respectively.

For most populations, bacterial concentrations obtained in gel beads from F1 (13 and 10 g L^{-1} of total carbohydrates) and F2 (10 g L^{-1} of total carbohydrates) were of the same order as those observed in fecal samples with differences below 0.9 Log10 CFU g^{-1} (Table 1). The exceptions were clostridia (plus 1.7 Log10) in F2 and Gram-positive cocci (plus 1.4 or 0.7 Log10 for F1 and 13 or 10 g L^{-1}),

Table 1. Bacterial populations measured by plate counts in feces and beads after immobilization and colonization for two fermentation experiments and different carbohydrate concentrations in the medium, and compared with *in vivo* ranges reported for infant feces

		Feces		Beads				
		Inocula		After immobilization†		After colonization‡		
	Range *in vivo**	F1	F2	F1	F2	F1 (13 g L^{-1})	F1 (10 g L^{-1})	F2 (10 g L^{-1})
	Log10 CFU g^{-1} wet feces	Log10 CFU g^{-1} wet feces		Log10 CFU g^{-1} feces		Log10 CFU g^{-1} beads		
Total anaerobes	8.5–10.5	10.4	10.4	9.5	8.9	10.4	9.6	10.0
Facultative anaerobes		9.2	9.4	8.5	8.1	9.7	8.7	9.8
Bifidobacteria	8.1–10.3	9.5	10.3	8.9	8.8	9.0	8.6	9.4
Bacteroides	7.4–9.8	9.6	8.9	7.6	7.7	9.9	8.8	9.2
Coliforms	7.4–9.5	8.9	9.3	7.9	7.9	9.6	8.5	9.7
Lactobacilli	<4.9	<4	8.2	<4	6.0	5.3	5.8	8.0
Clostridia	<4–7.1	8.1	6.7	7.3	6.0	6.4	7.3	8.5
Gram-positive cocci	7.7–9.4	7.9	9.1	7.5	7.8	9.3	8.6	9.7
Staphylococci	<4–7.3	5.0	7.5	<4	7.0	9.4	6.9	7.7

*Fecal populations of formula fed infants in early weaning period reported by Guerin-Danan *et al*. (1997) and Mountzouris *et al*. (2002).
†Bacterial concentrations in freshly prepared beads were expressed with respect to wet fecal samples used to inoculate (2% fecal inoculum with a fecal concentration of 20%) the polymer in the immobilization process.
‡Bacteria were enumerated in beads at days 14 and 29 for F1 with 13 and 10 g L^{-1} total carbohydrate respectively, and day 11 for F2.

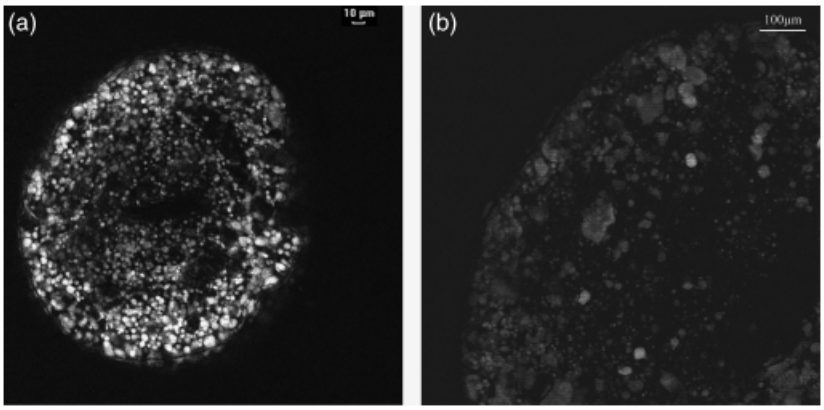

Fig. 1. Confocal laser-scanning microscopy micrographs of gel beads: total bacteria detected with Eub338 Cy3-labeled probe (a) and bifidobacteria detected with Bif164 Cy3-labeled probe (b) in beads collected during F1 after 14 days culture.

staphylococci (plus 4.4 or 1.9 Log10 for F1 and 13 or 10 g L^{-1}, respectively) and clostridia (minus 1.7 Log10 for F1 13 g L^{-1}). In addition, lactobacilli that were below the detection threshold (4 Log10 CFU g^{-1} feces) in the fecal sample used in F1 were recovered in beads at concentrations of 5.3–5.8 Log10 CFU g^{-1} beads for 13 and 10 g L^{-1} carbohydrates, respectively.

Fluorescence *in situ* hybridization was used to visualize the bacterial distribution in beads. Microscopy images of total bacteria (Eub338) and bifidobacteria (Bif164) in beads at the last day (d14) of the pseudo-steady state of F1 (13 g L^{-1}) are shown in Fig. 1. These images confirm the high colonization of beads by fecal bacteria which grew all over the bead volume, resulting in a broad distribution of total bacteria and bifidobacteria. Moreover, colonies tested with both Eub338 and Bif164 probes were larger at the periphery compared with the center of beads. No fluorescent signals were detected with Bac303 (*Bacteroides–Prevotella* cluster), Lab158 (*Lactobacillus–Enterococcus*) or Erec482 (*Clostridium coccoides–Eubacterium rectale* group).

Bacterial stability during chemostat cultures

The stability of the microbiota was evaluated from daily microbial analysis of fermentation effluents from the two continuous intestinal cultures, F1 (13 or 10 g L^{-1} of total carbohydrates) and F2 (10 g L^{-1} of total carbohydrates). Figure 2 shows the stability for the major bacterial populations in effluent analyzed by plate counts in R1 (PCS) and R3 (DCS) during the initial F2 stabilization period. Similar stability data were observed for R2 (TCS) and for the three reactors during F1 (data not shown). In R1, the pseudo-steady state was reached on day 4 for facultative anaerobe populations and F2, whereas the *Bacteroides fragilis* group

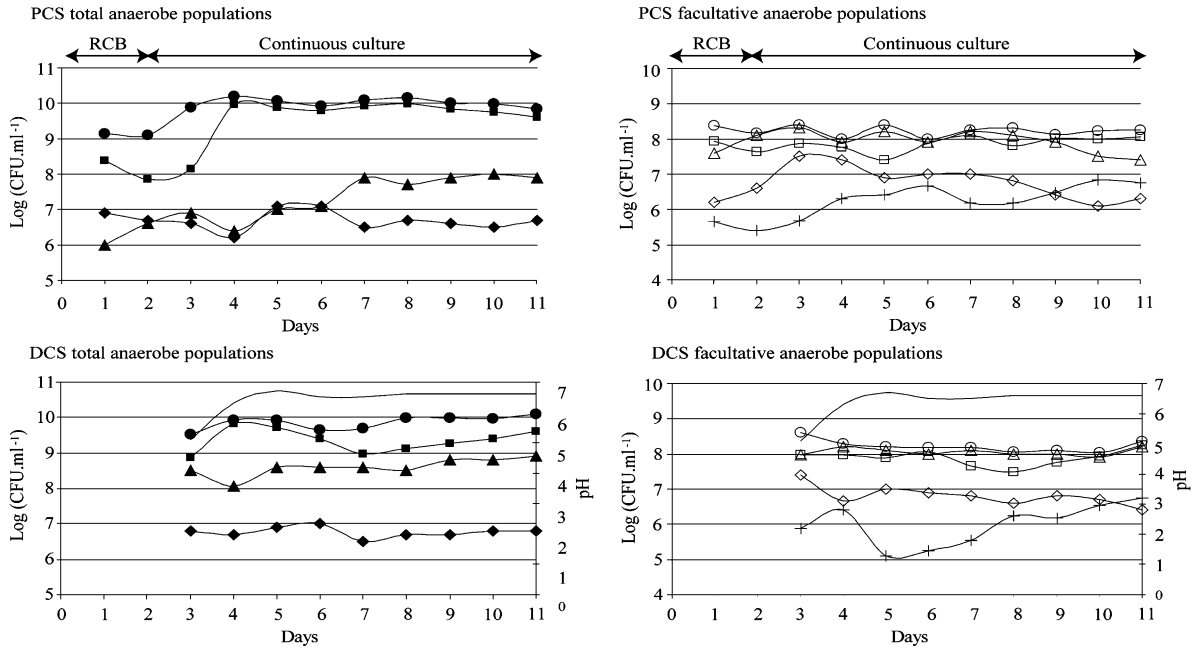

Fig. 2. Viable bacterial counts of total and facultative anaerobe populations in proximal (PCS) and distal (DCS) effluent from fermentation F2 with total carbohydrate concentration of 10 g L^{-1}: total anaerobes (—●—), bifidobacteria (—■—), bacteroides (—▲—), clostridia (—◆—), facultative anaerobes (—○—), Gram+cocci (—□—), staphylococci (—+—), coliforms (—△—), lactobacilli (—◇—), pH (···). RBC (repeated batch cultures) corresponding to beads precolonization.

took *ca*. 1 week to stabilize. Pseudo-steady states were generally obtained between days 5 and 7 for TCS, and days 5 and 10 for DCS depending on the population tested. For F1, similar results were obtained, with pseudo-steady states reached between days 6 and 7 in PCS, and on days 7 and 8 in TCS and DCS, respectively. Lactobacilli and staphylococci showed more variations (0.4–0.7 Log10 and 0.5–0.6 Log10 for PCS and DCS after days 7 and 8, respectively) during the pseudo-steady state than other populations in the intestinal model (*ca*. from 0.2 to 0.5 Log10).

Bacterial diversity in the intestinal model and comparison with fecal samples

Bacterial counts measured with selective media and FISH in fermented medium samples from all three reactors during pseudo-steady-state periods for F1 (10 and 13 g L^{-1} of total carbohydrates) and F2 (10 g L^{-1} of total carbohydrates) and their corresponding fecal inocula are reported in Table 2. In most cases, significantly higher counts were obtained with FISH compared with plate counts for both fecal and fermentation samples. In fecal and fermentation samples, Eub338 hybridized *ca*. 80% of the DAPI-stained bacteria, which were both used to measure total counts. The five different probes used in this study recovered 81%–99% of the total population measured with Eub338. All bacterial populations tested were detected with FISH, except the *Clostridium coccoides–Eubacterium rectale* group (probe Erec482), which was not detected in both fecal inoculum and effluent samples from the second fermentation (F2). Both detection methods (FISH and plate counts) showed that bacterial populations in the bulk liquid medium remained very stable among the three reactors (Table 2, data not shown for PCS and TCS in F1, 10 g L^{-1} total carbohydrate). The only exception was *Bacteroides* spp., for which bacterial counts increased from PCS to TCS or DCS as observed with FISH in both fermentations or with plate counts in F2.

Carbohydrate concentration (10 and 13 g L^{-1}) in the medium induced either small or no changes on the main bacterial populations in the effluents from DCS for F1, except for coliforms and staphylococci, which respectively decreased and increased by 0.8 Log10 (Table 2).

The bacterial profiles in DCS were compared with those in feces. There was no significant difference in total anaerobe numbers between DCS and fecal inoculum measured with plate counts (10.4 vs. 10.0 Log10 CFU mL^{-1}; $P > 0.05$) (Table 2). The counts in DCS effluent and fecal inoculum measured with DAPI were very close but significantly different (11.0 vs. 10.7 Log10 number of cell per mL; $P < 0.05$); the Tukey–Kramer-HSD test might have been too sensitive for these data. In both fecal inocula, facultative anaerobes and coliforms were more than one Log10 unit higher than in DCS from F1 (13 and 10 g L^{-1} carbohydrates)

Table 2. Bacterial populations measured by plate counts and fluorescence *in situ* hybridization in effluent samples from the three reactor stages at the pseudo-steady state and in fecal inocula for fermentation F1 (13 and 10 g L^{-1} of carbohydrate concentrations) and F2 (10 g L^{-1} of carbohydrate concentrations)

	Bacterial counts (Log10 CFU or cell number per mL medium or g^{-1} feces)*†									
	PCS (R1)		TCS (R2)		DCS (R3)				Feces	
	13 g L^{-1}		13 g L^{-1}		13 g L^{-1}		10 g L^{-1}			
Bacteria/probes	PC	FISH	PC	FISH	PC	FISH	PC	FISH	PC	FISH
Fermentation 1										
DAPI		10.7^{a}		10.7^{a}		10.7^{a}		nd		nd
Total anaerobes/Eub338	9.8_{A}	10.6^{a*}	10.2_{A}	10.6^{a}	10.0_{A}	10.6^{a*}	9.8	nd	10.4_{A}	nd
Facultative anaerobes	7.9_{A}		8.1_{A}		8.0_{A}		8.0	nd	9.2_{B}	
Bifidobacteria/Bif164	9.4_{A}	10.6^{a*}	9.6_{A}	10.4^{a*}	9.3_{A}	10.4^{a**}	9.3	nd	9.5_{A}	nd
Bacteroides/Bac303	7.9_{A}	9.2^{a*}	8.2_{A}	9.7^{a*}	7.8_{A}	9.8^{b*}	8.1	nd	9.6_{B}	nd
Coliforms/EC1531	7.8_{A}	7.5^{a}	8.1_{A}	7.4^{a*}	7.8_{A}	7.2^{b**}	7.0	nd	8.9_{B}	nd
Lactobacilli/Lab164	5.2_{A}	9.1^{a**}	5.6_{A}	9.1^{a**}	5.5_{A}	9.0^{a**}	5.8	nd	$< 4_{B}$	nd
Clostridia/Erec482	7.5_{A}	8.7^{a**}	7.5_{A}	8.6^{a**}	7.5_{A}	8.8^{a*}	7.6	nd	8.1_{B}	nd
Gram positive cocci	7.9_{A}		8.1_{A}		7.9_{A}		7.8		7.9_{A}	
Staphylococci	7.5_{A}		7.9_{A}		5.9_{B}		6.7		5.0_{C}	
	PCS		TCS		DCS					
	10 g L^{-1}		10 g L^{-1}		10 g L^{-1}				Feces	
	PC	FISH	PC	FISH	PC	FISH			PC	FISH
Fermentation 2										
DAPI		10.6^{a}		10.7^{a}		10.7^{a}				11.0^{b}
Total anaerobes/Eub338	10.0_{A}	10.5^{a*}	10.1_{A}	10.5^{a}	10.0_{A}	10.5^{a**}			10.4_{A}	10.9^{b*}
Facultative anaerobes	8.2_{A}		8.3_{A}		8.1_{A}				9.4_{B}	
Bifidobacteria/Bif164	9.8_{AB}	10.3^{a*}	9.4_{B}	10.4^{a*}	9.3_{B}	10.4^{a**}			10.3_{A}	10.8^{a*}
Bacteroides/Bac303	7.9_{A}	9.1^{a*}	8.7_{B}	9.4^{ab*}	8.8_{B}	9.6^{b**}			8.9_{B}	9.9^{b**}
Coliforms/EC1531	7.7_{A}	9.2^{a**}	8.1_{A}	9.2^{a*}	8.1_{A}	9.2^{a**}			9.3_{B}	9.1^{a}
Lactobacilli/Lab158	6.4_{A}	9.1^{a***}	6.5_{A}	9.2^{a***}	6.6_{A}	9.1^{a***}			8.2_{B}	9.5^{b*}
Clostridia/Erec482	6.6_{A}	ND	6.8_{A}	ND	6.7_{A}	ND			6.7_{A}	ND
Gram positive cocci	8.0_{A}		7.8_{A}		7.9_{A}				9.1_{B}	
Staphylococci	6.6_{A}		6.2_{A}		6.4_{A}				7.5_{B}	

Data are mean values calculated for the last 4 and 2 days for each fermentation period for plate counts and FISH, respectively.
PCS, proximal colon simulation; TCS, transverse colon simulation; DCS, distal colon simulation; PC, plate counts; nd, not determined; ND, not detected, value below the detection threshold of the FISH method which was 6.8 Log10 cell number per mL for effluent samples and 7.5 Log10 cell number per g for wet feces.
[a]FISH counts significantly different from plate counts at the significance level: $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.
[b]Effects of fermentation section (PCS, TCS and DCS) on bacterial populations: values with different letters (capitals for plate counts and small letters for FISH) are significantly different by Tukey–Kramer HSD test ($P < 0.05$).

and F2. Interestingly, the other bacterial populations behaved differently in F1 and F2 inoculated with different feces**.** *Bacteroides* and clostridia, present in high numbers in feces from baby 1, were significantly lower in DCS during F1 (13 and 10 g L^{-1} of carbohydrates), whereas the reverse was observed for lactobacilli and staphylococci. Bifidobacteria, lactobacilli, Gram-positive cocci and staphylococci present in high number in feces from baby 2 were lower in DCS from F2 whereas bacteroides and clostridia were not different. Effluent samples from DCS in F1 and F2 (total carbohydrate concentration of 10 g L^{-1}) showed more similar bacterial profiles, with population variations below 0.9 Log10 unit, except for coliforms (1.1 Log10), whereas the corresponding feces exhibited larger differences (Table 2).

Denaturing gradient gel electrophoresis profiles from feces (baby 2) and effluent samples of PCS, TCS and DCS from F2 during the pseudo-steady state were compared (Fig. 3). Bacterial profiles from fecal and fermentation samples showed high similarities, indicating that the complexity and diversity of the inoculum was apparently well conserved in the intestinal model. Most of the bands of the fecal inoculum were detected in fermentation samples. However, some bands detected in fermentation samples were not seen in fecal sample (Fig. 3, bands a and b), and

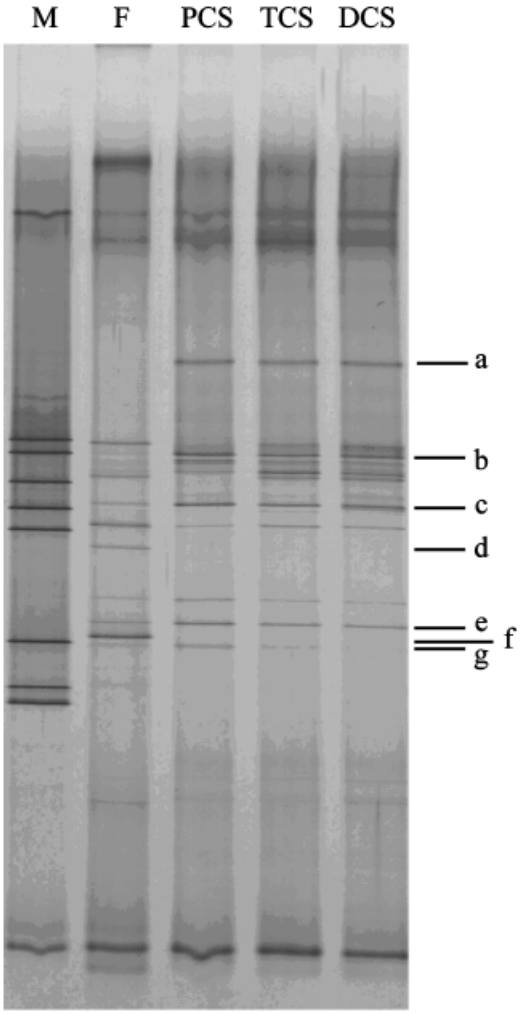

Fig. 3. PCR-denaturing gradient gel electrophoresis profiles showing the bacterial diversity in fecal inoculum (F) and in proximal (PCS), transverse (TCS) and distal (DCS) effluent samples from fermentation F2 with total carbohydrate concentration of $10\,g\,L^{-1}$. Bands detected in fermentation samples only (a and b), in fecal sample only (d and f), in fecal sample, PCS, TCS, and DCS with differences in intensity (c, e, g). Markers are shown in column M.

the reverse was also observed (Fig. 3, bands d and f). Some bands present in both fecal and effluent samples showed differences in intensity (Fig. 3, bands c, e and g).

Metabolite analysis

Short-chain fatty acids, isoacids, lactate and ammonia concentrations were analyzed in fecal and fermentation samples from the three reactors at the end of the stabilization periods (Table 3). In both experiments, total SCFA, isoacids and ammonia concentrations gradually increased from PCS to DCS with significant differences among reactors. Among SCFA, propionic acid for F1 ($13\,g\,L^{-1}$), and both propionic and butyric acids for F1 ($10\,g\,L^{-1}$) and F2, increased to a greater extent compared with acetate, inducing significant changes in SCFA ratios for TCS and DCS compared with PCS. For F1, the fermentation medium with $13\,g\,L^{-1}$ total carbohydrate induced a higher production of SCFA and iso-acids ($P < 0.05$) compared with the $10\,g\,L^{-1}$ medium. Very different SCFA ratios were measured for F1 (10 and $13\,g\,L^{-1}$) compared with F2, with a much lower butyrate proportion (5–8 vs. 13–19%) in F2. Moreover, isovaleric acid accounted for 22 to 33% of the iso-acids production in F1 (10 and $13\,g\,L^{-1}$), whereas no iso-valeric acid was detected during F2. There was no valeric acid, in the different samples tested. Lactic acid was only detected in PCS, at low concentrations of 2.8 and 0.8 mM for F1 ($13\,g\,L^{-1}$) and F2 ($10\,g\,L^{-1}$), respectively. For both fermentations and total carbohydrate concentration in the medium for F1, ammonia concentration largely increased from PCS to DCS ($P < 0.0005$, Table 3). In F1, the reduction of carbohydrate concentration from 13 to $10\,g\,L^{-1}$ induced a significant increase of ammonia concentration ($P < 0.0005$) in DCS, from 26.3 to 29.1 mM, but no effect was observed in PCS and TCS.

Metabolite concentrations in DCS for F2 were compared with those in feces. About twofold lower concentrations of total and individual SCFA were measured in feces. However, SCFA ratios in feces were very similar to those in effluent samples from DCS.

Discussion

In a previous study, we showed that the immobilization of fecal microbiota in gel beads allowed the preservation of the main bacterial populations as tested by the plate count method (Cinquin *et al.*, 2004). A single-stage chemostat, inoculated with immobilized fecal microbiota and operated under conditions reproducing those encountered in different sections of the infant colon, showed very good stability over 7.5 weeks of fermentation (Cinquin *et al.*, 2004). Beads were highly colonized by the major bacterial populations found in infant feces. In the present work, we used the same immobilization technique to model the entire infant colon, using a three-stage chemostat system in which beads retained in the first reactor (PCS) were used to inoculate the fermentation medium and two subsequent stages of the system.

Inocula used in F1 and F2 came from two different babies. Baby 2 (F2) had a typical microbiota of a breast-fed infant with higher counts of bifidobacteria and lactobacilli and lower counts of clostridia and *Bacteroides* spp. compared with baby 1, who had a bacterial profile more similar to that of a formula-fed infant (Stark & Lee, 1982; Harmsen *et al.*, 2000). The immobilization of fecal inocula induced a transient decrease in bacterial populations, which rapidly

Table 3. Metabolite concentrations and ratios in effluent samples from the three reactor stages at the pseudo-steady state and fecal inocula for fermentation F1 (13 and 10 g L^{-1} of carbohydrates) and F2 (10 g L^{-1} of carbohydrates)

	PCS (R1)		TCS (R2)		DCS (R3)		Fecal samples		
Fermentation metabolites[ab]	mM	%	mM	%	mM	%	mmol kg^{-1} wet weight feces	%	Pooled SEM[c]
Fermentation F1									
13 g L^{-1}									
Total SCFA	136.2[a]	100	167.4[a]	100	187.6[b]	100	nd		7.3
Acetic acid	94.3[a]	69	107.4[ab]	64	118.7[b]	63	nd		5.0
Propionic acid	23.9[a]	18	35.0[b]	21	42.2[b]	23	nd		2.4
Butyric acid	18.0[a]	13	25.0[a]	15	26.7[a]	14	nd		2.5
Isoacids	9.9[a]	100	18.2[b]	100	21.7[c]	100	nd		1.7
Isobutyric acid	7.7[a]	78	14.2[b]	78	17.3[c]	80	nd		1.7
Isovaleric acid	2.2[a]	22	4.0[b]	22	4.4[b]	20	nd		2.2
Ammonia	7.7[a]		24.6[b]		26.3[b]		nd		1.6
10 g L^{-1}									
Total SCFA	108.8[a]*	100	136.4[a]	100	146.1[a]**	100			
Acetic acid	77.7[a]*	72	84.4[a]*	62	94.0[a]*	65			4.3
Propionic acid	16.4[a]*	15	26.0[b]*	19	25.2[b]**	17			2.8
Butyric acid	14.7[a]	13	26.0[b]	19	26.9[b]	18			2.8
Isoacids	10.3[a]	100	17.5[a]	100	15.8[a]**	100			6.7
Isobutyric* acid	7.8[a]	76	11.1[a]	63	10.6[a]**	67			2.5
Isovaleric acid	2.5[a]	24	6.4[a]*	37	5.2[a]	33			2.0
Ammonia	10.2[a]		24.5[b]*		29.1[c]*				1.6
Fermentation F2									
10 g L^{-1}									
Total SCFA	118.6[a]	100	141.4[b]	100	157.3[c]	100	71.5 d	100	1.9
Acetic acid	92.7[a]	78	104.4[ab]	74	115.3[b]	73	54.0[c]	75	2.3
Propionic acid	20.4[a]	17	25.5[ab]	18	29.0[b]	19	13.5[a]	19	1.8
Butyric acid	5.5[a]	5	11.5[b]	8	13.0[b]	8	4.0[a]	6	1.3
Isoacids	3.6[a]		10.2[b]		12.6[b]		ND		0.9
Isobutyric acid	3.6[a]		10.2[b]		12.6[b]		ND		0.9
Isovaleric acid	ND		ND		ND		ND		
Ammonia	9.1[a]		29.4[b]		33.5[c]		29.1[b]		1.4

Data are means values calculated for the last 4 days of each period. Effect of fermentation section on metabolite production: values with different letters are significantly different with Tukey–Kramer HSD test ($P < 0.05$). PCS, proximal colon simulation; TCS, transverse colon simulation; DCS, distal colon simulation; PC, plate counts; nd, not determined; ND, not detected, value below the detection threshold of the HPLC method (2 mM).

[a]SCFA concentrations in F1 (10 g L^{-1} total carbohydrate) significantly different from SCFA concentration in F1 (13 g L^{-1} total carbohydrate) at the significance level: *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

[b]Effects of fermentation section (PCS, TCS and DCS) on metabolites concentrations: values with different letters are significantly different by Tukey–Kramer HSD test ($P < 0.05$).

[c]Pooled SEM, pooled standard errors of the mean.

increased during repeated-cycle batch process cultures and first days of continuous culture. In F2, bacterial counts in beads at the pseudo steady-state were very close to those in feces (baby 2), except for bifidobacteria and clostridia, which were present in higher and lower numbers in feces than in beads, respectively. Larger differences were observed for F1 (13 and 10 g L^{-1} carbohydrates), with lactobacilli and staphylococci counts being higher in beads than in fecal sample, which showed very low concentrations of these two populations, while clostridia counts were lower in beads. However, bacterial counts in beads from F1 operated at 13 g L^{-1} of total carbohydrates were closer to those in feces compared with F1 operated at 10 g L^{-1}. Indeed, a reduction of carbohydrate concentration from 13 to 10 g L^{-1} in F1 had a major impact on immobilized bacterial populations, showing the influence of fermentation conditions on the microbial balance established in beads. Bacterial counts in beads from F1 (13 or 10 g L^{-1}) and F2 were in the range of bacterial counts measured *in vivo* (Table 1).

Immobilized bacteria were also analyzed with FISH and confocal microscopy (Fig. 1). Micrographs with Eub338 and Bif164 hybridization, the only probes allowing good bacterial detections, showed that all the volume of the bead was colonized. This growth pattern is different from

colonization of beads with pure cultures of bifidobacteria or lactic acid bacteria where a very sharp cell concentration gradient was observed; bacterial growth occurred mainly in a thin (150–250 μm) peripheral layer in beads, largely due to the sharp pH gradient (Doleyres *et al.*, 2002). This complete colonization of gel volume by fecal microbiota may be explained by different pH gradients in beads where production of organic acids mainly by bifidobacteria could be balanced by local consumption of these acids by other bacteria, resulting in a reduced or absent pH gradient. *Bacteroides* could not be visualized due to the small size of their colonies, whereas bacteria targeted with Lab158 and Erec482 were present in low numbers which could not be detected by this method.

For both fermentation experiments, high total cell concentrations were tested in the reactor effluents with DAPI or Eub338 (*ca.* 10.7 Log10 number of cell per mL) and plate counts (*ca.* 10.0 Log 10 CFU mL^{-1}). These cell concentrations are generally higher than data generally reported for free cell continuous culture systems, with DAPI and plate counts' total bacteria concentrations of 9.5 Log10 number of cell per mL or 9.3 Log10 CFU mL^{-1}, respectively (Probert *et al.*, 2004; Possemiers *et al.*, 2004). These results confirm that cell immobilization can lead to high and stable cell density in both beads and liquid medium, equivalent to that observed in feces (Cinquin *et al.*, 2004). As already observed *in vitro* (De Boever *et al.*, 2001) as well as *in vivo* (Khaddour *et al.*, 1998), bacterial populations were very stable between the different reactors simulating the different colonic segments. One exception was *Bacteroides* spp., where FISH counts increased from PCS to DCS in F1 and F2 (Table 2). This phenomenon might be related to the ability of *Bacteroides* spp. to compete more effectively under conditions of limited substrate availability as occurred in DCS (Hopkins & Macfarlane, 2000; Cinquin *et al.*, 2004). Indeed, Kuwahara *et al.* (2004) showed that *Bacteroides* spp. contain more polysaccharide-degrading enzymes with a wide range of substrate specificities compared with bifidobacteria. Lactobacilli and staphylococci counts exhibited high variations compared with other bacterial populations, which has also been observed by Possemiers *et al.* (2004) during continuous colonic fermentation with free cells. Differences in bacterial concentrations observed between FISH and plate counts methods (Table 2) can be attributed to differences in targeted populations for selective media and oligonucleotide probes, and accuracy of the methods (Harmsen & Welling, 2002). This is the case for Lab154, which detected both lactobacilli and enterococci and measured significantly higher cell concentrations than the lactobacilli LAMVAB selective medium used in this study.

As already noted for beads, although the microbial complexity of inocula was maintained in the immobilized cell colonic model, profiles of bacterial populations in DCS effluent samples from both fermentations did not closely reproduce the bacterial balance in the inocula. In general, bacterial populations present in high numbers in fecal samples tended to decrease in DCS, for example, bifidobacteria and lactobacilli numbers decreased, respectively, by 1 and 1.6 Log10 between inoculum and DCS in F2. In contrast, bacteria with lower numbers in feces tended to increase during fermentation, as illustrated for lactobacilli whose numbers in F1 inoculum were below the detection limit but were detected in fermentation samples. Similarly, staphylococci numbers increased by 1.7 Log10 between inoculum and DCS in F1 (Table 2). As a consequence, large differences in bacterial numbers observed between inocula used in F1 and F2 were attenuated in DCS effluent samples from the fermentations. Our microbial data suggest that the colonic system with immobilized cells produces a more standard bacterial equilibrium from different inocula. This effect could be explained by the standard culture conditions, such as retention time, pH and medium used in the chemostat model which led to a bacterial equilibrium partially controlled by these conditions, and eventually minimizing the high variability found in bacterial composition of feces (Mountzouris *et al.*, 2002). This characteristic of the immobilized cell model could be advantageous for experimentation of intestinal fermentation in terms of decreasing the variability associated with large changes in microbiota composition and increasing repeatability of experiments and validity of data.

The different bacterial populations in DCS from both fermentations reached concentrations in the same range as those usually recovered in infant feces (Table 1) (Kirjavainen *et al.*, 2002; Mountzouris *et al.*, 2002). Moreover, the comparison of DGGE profiles between fecal and fermentation samples showed complex patterns corresponding to bacterial diversity found in infant feces (Favier *et al.*, 2002). The high number of bands, together with the preservation of the major bands in fermentation effluents, suggests that bacterial diversity of the inoculum was well preserved by the immobilized cell system. Our population and DGGE data suggest that a diversified bacterial equilibrium in agreement with *in vivo* data was established in the fermentation system.

Bacterial metabolism was largely influenced by the availability of carbohydrates in the fermentation medium, as previously reported by Macfarlane *et al.* (1998b), whereas bacterial counts in the broth medium were either not or little affected (Table 2 and 3). In fermentation F1, a reduction from 13 to 10 g L^{-1} of total carbohydrates led to a decrease in SCFA production and increase in ammonia for all reactors. However, the smaller amount of butyrate observed in all reactors during F2 compared with F1, for both 13 and 10 g L^{-1} total carbohydrate, was not related to a decrease in available polysaccharides in F2, since butyrate concentration and ratio were little affected by

polysaccharide concentration during F1 (Table 3). This data can be explained by the absence of bacteria belonging to the *Clostridium coccoides–Eubacterium rectale* group as detected by Erec482 and FISH, which contains most of the butyrate producers in human feces (Barcenilla *et al.*, 2000). Indeed, butyrate production was shown to be very low in breast-fed babies, as shown for baby 2 (F2), compared with formula-fed babies (Edwards *et al.*, 1994).

For both fermentations, carbohydrate breakdown occurred mainly in PCS, resulting in an important SCFA concentration, which only increased by 38% and 33% from PCS to DCS with 13 g L^{-1} (F1) and 10 g L^{-1} (F1 and F2) carbohydrate concentrations, respectively. This accumulation of SCFA in the medium (Table 3) has already been observed in continuous *in vitro* models where there was no SCFA absorption by epithelial cells (De Boever *et al.*, 2001). The increase in propionate concentration in TCS and DCS compared with PCS for both experiments can be related to an increase of *Bacteroides* spp. (Table 2), one of the main propionate producers of the intestinal microbiota (Blottiere *et al.*, 1999), as already observed by Cinquin *et al.* (2004). Lactate was only detected in PCS at very low concentrations for both fermentations. Lactate is an intermediate metabolite which is used by many bacteria (Seeliger *et al.*, 2002), and does generally not accumulate in the intestine. Ammonia and isoacids concentrations also increased by 3.4- and 2.2-fold from PCS to DCS with 13 g L^{-1} of carbohydrate (F1) and by 3.7- and 3.5-fold with 10 g L^{-1} of carbohydrate (F2), respectively. The dramatic increase compared with SCFA from PCS to DCS can be partly explained by accumulation of metabolites in the medium but also by the elevation of proteolysis usually observed towards the end of the gastrointestinal tract (Seeliger *et al.*, 2002). For F2, SCFA concentration was about twofold higher in DCS than in the inoculum, probably due to the absence of SCFA absorption in the model system. However, the relative ratios of SCFA in effluent samples from DCS were very similar to those in the inoculum and in the range of published data for fresh feces (Mountzouris *et al.*, 2002). Moreover, the difference in isoacid concentrations between fecal inoculum (no detection) and DCS in F2 could be a reflection of the different composition in precursor amino-acids, leucine, isoleucine and valine in the intestinal chyme of baby 2 and fermentation medium (Macfarlane & Macfarlane, 1997).

In conclusion, bacteriological and metabolic data from the three-stage infant colon chemostat model with immobilized fecal microbiota showed that complex microbiota were established and maintained at high concentrations in all three vessels. Both fermentation media tested were able to sustain high cell density and metabolic activity in the fermentation system. A bacterial balance that was more dependent on fermentation conditions than bacterial composition of the inocula was produced for the main bacterial groups. This bacterial community, as well as its metabolism, reflected those tested in infant feces. Moreover, our data indicate a good response of microbiota to culture conditions, such as carbohydrate substrate concentration. The three-stage *in vitro* colon model with immobilized cells could be a useful tool for studying bacterial communities and metabolism, and also for testing the effects of various environmental parameters on infant or adult microbiota. Immobilized fecal microbiota could also be used to improve stability and validity of the intestinal compartment in more complex digestive models that simulate digestive steps, colonic fermentation and absorption.

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